EFFECTS OF AGING UPON GERM CELLS AND UPON EARLY DEVELOPMENT.

PART II., CHANGES IN MODERATELY AGED EGGS AND SPERM.

A. J. GOLDFARB,

COLLEGE OF THE CITY OF NEW YORK.

CONTENTS.

In a previous publication (Goldfarb, '17), the writer determined the variation of freshly liberated sperm and eggs from freshly collected sea urchins of three different species. Two of these, *Toxopneustes* and *Hipponoë*, inhabit the shallow tropical waters of the Dry Tortugas, and the third, *Arbacia*, is common in the deeper colder waters near Woods Hole, Mass. In each species preliminary experiments had been made to ascertain the optimum conditions of development, such as volume and surface and filtration of sea water, concentration of eggs and sperm, etc., and such optimum conditions were used in all subsequent experimentation.

It was shown that even under these constant and optimum conditions, the freshly liberated eggs of different females varied in respect to (1) size, (2) jelly layer, (3) membrane formation, and (4) cleavage; that there was a surprisingly large variation, with respect to each of these four classes, in the eggs of different females; that the sperm of different males also varied, but to a much less degree. And finally the range of variation was determined for each species.

It was suggested that these variations served as convenient and accurate indices of the physiologic condition or vitality of the freshly removed eggs or sperm of different individuals.

In the present paper the same technique, the same three species of sea urchin, the same nomenclature and the same precautions were taken. It is proposed to determine first the physiologic condition of the germ cells, at the time of liberation, from given individuals, and then to determine the nature and the extent of the changes in the eggs and sperm as they become increasingly "overripe," "aged" or" stale."[1] In the next study, Part III., it is proposed to study other changes that appear in very late stages of overripening or ageing of the germ cells. I reserve for Part III. a discussion of the entire problem, and the bibliography.

The changes that were observed in moderately aging eggs, and those which will be discussed in detail are: (1) size, (2) jelly layer, (3) membrane formation and (4) cleavage.

I wish to acknowledge my indebtedness to Dr. A. G. Mayer, Director of the Marine Biological Laboratory of the Carnegie Institution of Washington, and to Prof. F. R. Lillie, Director of the Marine Biological Laboratory at Woods Hole, Mass., for the opportunity of working on this problem at their respective laboratories, and for the many facilities offered in connection with this work.

Effect of Age Upon Size of Eggs.

Hipponoë and Arbacia.

The diameters of freshly removed unfertilized eggs of each freshly collected female, were measured with an ocular micrometer (no. 3 eye piece and 1/6 objective) and the usual precautions concerning pressure, focusing, sampling, etc., taken. At different intervals (ages) other samples of the eggs of the same female were measured. The data are brought together in Table I.

I have shown (Goldfarb, '17) that the normal size of freshly liberated eggs of *Hipponoë* varied from 18½ to 20 ocular units in diameter, with a mode of 19½ units. In the present series

[1] By age is meant the time since removal or extrusion of ripe germ cells.

TABLE I.

SHOWS CHANGE IN SIZE AND LOSS OF JELLY LAYER WITH AGE. SIZE IS GIVEN IN OCULAR MICROMETER UNITS OF THE DIAMETER OF THE EGGS AT DIFFERENT AGES. *Hipponoë.*

No.	Date.	Age of Eggs, Hrs.	Diameter of Eggs in Ocular Micrometer Units. Number of Eggs.									Per Cent. of Eggs with Jelly Layer.	Per Cent. Loss of Jelly per Hour.
			21.0.	20.5.	20.0.	19.5.	19.0.	18.5.	18.0.	17.5.	17.0.		
1	6/15	5				*	*						
		22	*	*	*								
2	6/23	5				1	8	1				97	
		29				4	7	1				78	0.7
3	6/28	½				1	7	4				100	
		18½				8	18	3				100	0.0
4	6/28	½			2	5	6	2				100	
		18½			3	8	5	0				94	0.3
5	6/25a	2			2	7	1					100	
		5		2	8	3	1					91	
		24		7	1	3	2					40	2.7
6	6/27a	3			1	12	3					96	
		9			12	5	1					96	
		27		1	9	2	0					79	0.7
		33		0	13	3	0						
7	6/26a	0		1	3	6	3					86	
		18		3	3	6	0					77	
		24				9	5	1				62	1.0
		40						1	9	6	5	0	
		47							1	9	2	0	
8	6/26b	0			8	3						95	
		18	2	4	8	4						95	
		24			7	3						72	0.9
9	6/26c	0				0	4	8				90	
		18				0	15	2				60	
		24				3	8	3				6	3.5
		40				3	12	1				2	
		47				1	6	6				1	
10	6/25b	2				1	9	1				63	
		5				1	10	1				1	
		24					5	7	4	1		0	2.8
11	6/27b	3	2	4	3	6	0	0				80	
		9			1	4	9	1				10	
		27			1	9	2	0				1	3.3
		33			1	2	3	0	2	2	1	0	
12	6/26c	0			5	10						99	
		18		2	12	2						98	
		24		0	12	1	1					98	0.4
		40		1	11	1	1					62	
		47		1	12	1	0					17	
Variation in freshly liberated eggs of different females...			2	6	34	72	54	16	0	0	0	90	

the same variations occurred in the freshly liberated germ cells. As the eggs aged, there was a clear, considerable and progressive change in size. The observations may best be considered in three groups.

Female 1, is an example of the first group. When her eggs were 5 hours old, *i. e.*, 5 hours after liberation, their diameters were 19 and 19.5 units. When her eggs were 22 hours old, they measured 20, 20.5 and 21 units, *a clear and sharp increase in minimum, mode and maximum diameters, with age.*

In female 2, observations were made when the eggs were 5 and 29 hours old. In this female there was also an enlargement but very small. In females 3 and 4, observations were made at ½ and 18 hours respectively. In each female the eggs enlarged with age. In female 5, three observations were made, viz., 2, 5 and 24 hours, with a progressive enlargement at each successive age. In female 6, observations were made at four intervals, 3, 9, 27 and 33 hours. As in female 5, there was at first a rapid enlargement of the eggs, then a slow increase, and, in very late aging, a rapid increase again. The average diameters at each of the four intervals was 19.44, 19.80, 19.77 and 19.96.

In all the eggs of this first group, there was a cumulative increase in volume, with age, for the first 33 hours.

The second group includes such females whose eggs were observed over a longer period than 33 hours. In this group as in the first, the eggs also enlarged at first, with age, but after a longer interval there occurred a secondary diminution in size, and in very old eggs became even smaller than the norm.

For example, the eggs of female 7 were measured at 0, 18, 24, 40 and 47 hours. When 18 hours old the eggs were slightly larger than at the first observation. At 24 hours, the eggs were clearly smaller, and at 40 hours smaller still. At 47 hours they were much below their original size and below the norm for the species. The maximum size had diminished from 20.5 to 18, the mode from 19.5 to 17.5, the minimum from 19 to 17.

In female 8, there was the same initial increase and subsequent diminution towards the norm, when 24 hours old. In female 9, observations were made at 0, 18, 24, 40 and 47 hours. There occurred the usual enlargement until the 40th hour, after which there was a definite diminution in volume.

In a third group, of a few females only, the eggs though freshly removed, were in poor physiologic condition. In this group *the initial enlargement did not take place.* In these eggs

there occurred a direct diminution with age. In this group belong females 10 and 11, and probably female 7.

It should be recalled that the physiologic condition of any set of eggs was not determined merely by egg size, but by the results of several tests, such as the jelly layer test, the membrane test as well as the cleavage test, etc. By all of these tests, it was clearly and definitely shown *that eggs in good physiologic condition at the time of liberation, increased steadily with age, within the limits shown in Table I., and subsequently diminished in size by a process of fragmentation described in Part III. Eggs in physiologically*

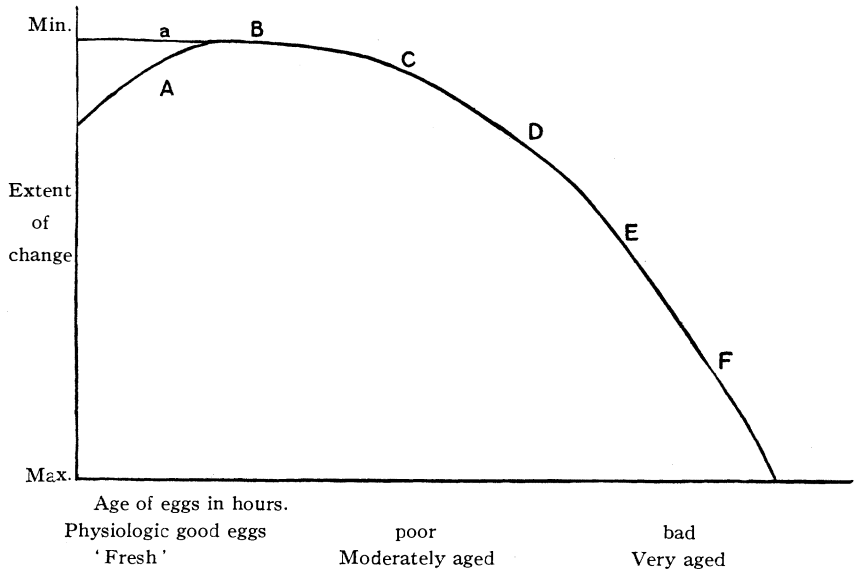

FIG. 1. Extent of morphologic and physiologic change in aging or deteriorating eggs. *A', B, C, D, E, F,* represents the change in volume, rate of membrane formation, rate of cleavage, total cleavage (*Hipponoë*). *A, B, C, D, E, F,* represents the change in width of fertilization membrane, loss of jelly, total cleavage, etc. *A', B* is period of superripening; *B, C, D,* the period of overripening or deterioration; *D, E, F,* the period of extreme deterioration or dying of the eggs.

poor condition at the time of liberation behaved like half-aged eggs, that is, they either enlarged but little, or did not enlarge at all, and subsequently diminished to and below the norm of the species.[1]

Figure 1 represents graphically and schematically the change

[1] So constant is the relation between volume and physiologic condition, tha one can predict the one from the other, with remarkable exactness.

in volume with age. Physiologically good eggs show an initial rapid increment, region *A* of the curve; then a progressively slower increment, region *B;* thirdly a progressive *diminution*, region *C*, towards *D* or even below the norm of the species to *E*. Eggs in moderately poor physiologic condition at liberation have already undergone physiologic deterioration, corresponding with part *A* within the body of the mother. Upon liberation they show only parts *B*, *C*, *D*, etc., of the curve. Eggs in very poor condition upon liberation have undergone changes represented by parts *A* and *B*, within the body of the mother, and upon liberation show only parts *C* and *D*, etc.

The significance of this increment with age lies in the changed permeability of the cortical layer which is denoted by increase in volume. This progressive increase in permeability permits increasing volumes of sea water to penetrate the egg and thus enlarge it. This process continues until partial cytolysis (fragmentation) reduces the size once again towards the norm, or until complete cytolysis destroyed the eggs altogether.

The change in size with age really measures the change in permeability of the cortical layer.

Similar observations were made upon *Arbacia* eggs, and the changes are so much in agreement with those of *Hipponoë* that it seems unnecessary to describe them here. There is the same initial increment followed by the secondary diminution with age. And the rate of change depended upon the physiologic condition of the eggs at the time of liberation and upon temperature. The two species differ in the markedly slower rate of change of *Arbacia*, due in largest part to the lower temperature of the sea water.

Effect of Age Upon the Jelly Layer.

Hipponoë and Arbacia.

The jelly layer of the same sea-urchin eggs were made clearly visible by adding Chinese black to the sea water, as suggested by F. R. Lillie. Samples of 150 to 200 consecutive freshly removed (and unfertilized) eggs of each female were examined, avoiding mechanical or chemical removal of the jelly, and the presence or absence of the jelly layer recorded. At successive intervals

other samples of the same female were examined, and the results reduced to percentages as recorded in Table I.

I have shown (Goldfarb, '17) that the freshly liberated eggs of different females vary considerably in the per cent. possessing the jelly layer. In *Hipponoë* the per cents vary from 100 to 63 per cent., in *Arbacia* from 100 to 59 per cent. These differences, I suggested, indicated corresponding differences in physiologic condition of the eggs at the time of liberation.

Whatever the condition of the eggs at the time of liberation, with increasing age there was a continuous decrease in the number of eggs with the jelly layer present.

The data may best be considered in two groups, according to the physiologic condition of the eggs at the time of the first observation.

In the first group are those females whose eggs were in good physiologic condition. This was indicated by the high per cent. (*i. e.*, above 95 per cent. with jelly layers) at the first observation, as well as by other tests. In this group are placed females 2, 3, 4, 5, 6, 8 and 12, and the corresponding percents of jelly layers was 97, 100, 100, 100, 96, 95 and 99 respectively.

In the second group are placed those females whose eggs were in poor physiologic condition. In this group are included females 7, 9, 10 and 11, whose corresponding jelly counts were, 86, 90, 63 and 80 per cent., respectively.

In both groups the surrounding jelly layer progressively disappeared with age, as seen in Table I. But the rate of loss was different. An example of the first group is female 8. When 0 hours old, 95 per cent. possessed the jelly layer, when 18 hours old 95 per cent., and when 24 hours old only 72 per cent. retained the layer. Female 11 is an example of the second group in which the loss was decidedly greater. The eggs when 3 hours old showed 80 per cent. with jelly layers, when 9 hours old but 10 per cent., when 27 hours old only 1 per cent., and when 33 hours old none of the eggs possessed the jelly envelope.

The two groups differ then, not only in original per cent. with jelly layers, but also in the rate of loss of this layer. In the first, containing physiologically good eggs, the loss of the jelly layer per hour, for the indicated intervals for the different females, was 0.7, 0.0,

0.3, 2.7, 0.7, 0.9, 0.4 per cent., with an average of 0.81 per cent. loss per hour. In the second group with physiologically poor eggs the loss was, 1.0, 3.5, 2.8, 3.3 per cent. per hour with an average of 2.65 per cent. or over three times as rapid. I cannot account for the only apparent exception, namely female 5, whose rate of loss was 2.7 per cent.

Fig. 1 represents the loss of jelly with age. With physiologically good eggs, the numbers with jelly is a maximum or nearly so, and the rate of loss with age is small, part *a* and *B*. As the eggs become physiologically poor, and in eggs which were in poor physiologic condition at the time of liberation, the numbers with jelly is much less, and the rate of loss with age is more rapid, parts *C*, *D*, etc. It should be noted in passing that parts *B*, *C*, *D* and *E*, are parallel with the corresponding parts of the curve showing change in the volume of eggs with age.

The observations upon *Arbacia* eggs are in entire accord with those of *Hipponoë*. They differ only in the slower rate of loss. One example may illustrate this slower rate. The eggs when freshly removed showed 90 per cent. with their jelly layers. When 41 hours old, 50 per cent. possessed the layer, when 46 hours, 19 per cent., when 70 hours, 1 per cent., and when 72 hours none of the eggs possessed the jelly layer. *In both species of eggs the loss of jelly was a function of age, and physiologic condition at time of the first observation.* If the loss of jelly is known, one can predict (provided proper precautions are taken) the physiologic condition of the eggs and vice versa, from the physiologic condition one may determine the loss of jelly.

Loeb, Harvey and F. R. Lillie in particular, as well as others, have recorded the loss of jelly in old eggs. My observations show not only a loss with age, which is different for different species, but I have determined the rate of loss with age. More than this, I have shown that the rate of loss depends not only upon the species and temperature, but upon the condition of the eggs at liberation, and finally that loss of jelly serves as another symptom of the physiologic condition or vitality of the eggs at any time, and that this vitality diminished at a known rate with age.

Effect of Age upon Membrane Formation.

A third sympton of aging and physiologic deterioration is the change in fertilization membrane. A number of investigators have observed that old eggs do not form fertilization membranes (Hertwig, R. and O., '86, Loeb, '03, '15, Harvey, '10, '14, F. R. Lillie, '14, etc.). Harvey ascertained the exact age in *Arbacia* beyond which, namely 52 hours, no membranes were formed. Just, '15, observed that the jelly layer, which follows fertilization in *Nereis* was retarded with aging.

Before considering in detail the changes in the fertilization membrane, with age, and the causes of such changes, it should be recalled that freshly liberated eggs of freshly collected females, when fertilized under constant and optimum conditions, varied considerably in the rate of membrane formation. Such freshly liberated eggs may be placed into three groups according to their physiologic condition, namely (1) those that form membranes within 2 minutes after fertilization, which are in good physiologic condition. (2) Those that form membranes in 3 to 7 minutes, and which are in moderately poor condition, and (3) those that do not form membranes, which are in poor physiologic condition. It should also be recalled that the rate of membrane formation is further complicated by the differential effect of eggs and sperm. I will mention but two of the experiments, which are typical, to illustrate this differential effect. In experiment 1, Table II., *the eggs of one female were fertilized by the sperm of 5 different males.* All the germ cells were 43 minutes old. Four of the males induced membrane formation in 1½ minutes, the fifth in 2 minutes. Even greater uniformity occurred when the germ cells were 1 hour old, for *the eggs of all 5 samples of the same female formed their membranes at the same time, namely 1 minute.* When the germ cells were 1½ and 2¼ hours old, the eggs again reacted in the same manner for none formed membranes.

In contrast to this uniformity of behavior, when the eggs of one female were fertilized by different males, are those experiments in which the eggs of different females were fertilized by the one male. For example, in experiment 2, the eggs of 3 females were

fertilized by the one male, when the germ cells were 20 minutes old. Fertilization membranes first appeared in the three groups of eggs as follows, 3½, 1½ and 1¾ minutes respectively. When the germ cells were 80 minutes old, they appeared in 2⅔, 1⅔, 1⅚ minutes; when 140 minutes old, 1⅔, 1½ and 1 minute; when 280 minutes, ⅔, 1¼ and 1 minute.

In the other experiments similar results were obtained, i. e., uniform or nearly uniform rate of membane formation, when the eggs of one female were fertilized by different sperm, and frequently;

TABLE II.*a*.

SHOWS CHANGE IN RATE OF MEMBRANE FORMATION WITH AGE, OF EGGS OF DIFFERENT FEMALES. *Toxopneustes.*

No.	Date.	Age of Germ Cells in Minutes.	Minutes Required Before first Membranes Appeared.						Average No. Minutes.
			Male 1.	2.	3.	4.	5.	6.	
1	7/3	43	2	1½	1½	1½	1½		1.6
		59	1	1	1	1	1		1.0
		89	N	N	N	N	N		N
		140	N	N	N	N	N		N
			Female 1.	2.	3.	4.	5.		
2	7/14	20	3½	1½	1¾				2.25
		80	2⅔	1⅔	1⅚				2.05
		140	1⅔	1½	1				1.39
		210	1¼	1¼	1¼				1.25
		280	⅔	1¼	1				0.97
		370–600	N	N	N				N
3	7/21	120	1½	1					1.25
		323	1⅓	1					1.15
		450	N	N					N
4	7/16	160	N	N	2½	2½	0	2½	2.5
		300	N	N	N	N	N	1½	0.5
		500–600	N	N	N	N	N	N	N
5	7/7	75	2	3	2½	3½			2.75
		84	1	2	1½	2			1.62
		180	2	2	4½	6			3.62
6	7/12	17	1⅔	1	1				1.22
		42	1½	1	1				1.16
		99	½	¾	¾				0.66
		161	⅓	1	⅔				0.66
		214	⅕	⅔	¾				0.53
		294	2½	⅚	N				1.11
		360	5½	N	N				1.83
		420	N	N	N				N
7	7/19	130	3	3	2	1½	1½	1½	2.08
		240	N	2	2	1⅓	1⅓	1½	1.36
		350	N	2	2	2	2	2	1.83
		470	N	3½	4	N	2½	2½	2.08

N = no membrane in 10 minutes.

wide differences when the eggs of different females were fertilized by the one male. There was a far greater variation among the eggs of different females than among the sperm of different males, or, putting the matter in a slightly different way, the extent of variation in development is conditioned much more by the eggs than by the sperm.

With these facts in mind, we may proceed with the examination of the effect of age upon the membrane formation in physiologically "good," "moderately poor" and "poor" eggs. The data are brought together in Tables II.*a* and II.*b*.

TABLE II.*b*.

SHOWS CHANGE IN RATE OF MEMBRANE FORMATION OF AGEING EGGS AND SPERM.

Arbacia.

No.	Date.	Age of Germ Cells in Hours.		Minutes Required Before Membranes Appeared.						Average No. Minutes.
		♀.	♂.	Female 1.	2.	3.	4.	5.	6.	
1	8/12	4	4	2	1½	3	2	2		2.1
		23	23	3	7	N	N	N		5.0
		28–48	28–48	N	N	N	N	N		N
2	8/14	4	4	2	2	2	2	2	2	2.0
		24	1	N	N	3½	2	N	N	2.7
		31	8	N	N	N	N	N	N	N
		49	1¼	N	N	N	N	N	N	N
		5	5	1½	1½	½	½	½		
3	8/16	1½	1½	1½	1½	1½	1½	1½	1½	1.5
		18½	18½	3½	N	3½	3½	3½	N	3.5
		23	23	N	N	N	N	N	N	N
		24	4	2½	N	2½	N	6	N	3.6
		42	1	N	N	N	N	N	N	N
		48–75	1/10–¾	N	N	N	N	N	N	N
4	8/18	1	23	7	3	3	3			
5	8/19	1	45½	2	N	2½				
6	8/20	5	73	2½	4	4½	5			
		10	95	2½	N	N	N			

N = no membranes in the first 10 minutes.

In the first group, *containing physiologically good eggs, the rate of membrane formation was accelerated with age.* For example in experiment 2, the eggs of the 3 females were fertilized by one male when the germ cells were 20, 80 140, 210, 280, 370, 440 and 550 minutes old. The eggs of female number 1, for instance, formed their membranes at the above ages in 3½, 2⅔, 1⅔, 1¼ and ⅔ minutes, and none after 370 minutes. The eggs of female number 3 formed their membranes in 1¾, 1⅚, 1,

1¼ and 1 minute and none after 370 minutes. The average for all 3 females at the give ages was 2.25, 2.05, 1.39, 1.25, 0.97 minutes, and none between 370 and 600 minutes when the experiment terminated. A corresponding acceleration in the rate of membrane formation, with age, occurred in all the other experiments with physiologically good eggs.

In the second group of physiologically poor eggs, there was also an acceleration in rate of membrane formation with age, but subsequent to this, there was a period of retardation, and ultimate inability to form membranes.

For example in Experiment 5, the eggs of 4 females were fertilized by the same male when the germ cells were 75, 84 and 180 minutes old. The eggs of female no. 1 formed their membranes at these ages in 2, 1 and 2 minutes respectively, in female 3, in 2½, 1½ and 4½ minutes, in female 4 in 3½, 2 and 6 minutes respectively. Female 2 alone did not show any retardation. The average for all 4 females was *2.75, 1.62 and 3.62 minutes respectively.*

In experiment 6, of the three females whose eggs showed an initial acceleration, only one showed a secondary retardation. The observations in this experiment were made at more intermediate ages namely, 17, 42, 99, 161, 214, 294, 360, 420 and 430 minutes respectively. The eggs of female no. 1 formed membranes in 1 ⅔, 1½, ½, ⅓, ⅕ minutes and formed no more membranes after 420 minutes. The average rate for all three females for the corresponding ages, was *1.22, 1.16, 0.66, 0.53, 1.11 and 1.83 minutes* respectively, and none after 420 minutes.

In experiment 7, 5 out of 6 females showed the initial acceleration with age, and the secondary retardation. The average for the 6 females for the following ages 130, 240, 350 and 470 minutes, was *2.08, 1.36, 1.83 and 2.08 minutes respectively.*

The results may be graphically represented as in Fig. 1. The ordinates represent the inverse time or rate of membrane formation. The abcissas represent age of germ cells. With physiologically good eggs, the time and rate of membrane formation decreases with age, parts *A* and *B* of the curve. Beyond certain limits when the eggs are in poor physiologic condition, the time and rate is correspondingly increased, parts *C*, *D*, *E*,

etc. In eggs, physiologically poor at the time of liberation, there is either a short period of acceleration, part *B*, followed by a long period of retardation, parts *C*, *D*, *E*, etc. In eggs in still poorer condition, the retardation takes place from the very beginning of the experiment, parts *C*, *D*, *E*, etc. Hence the better the condition of the eggs the longer the membrane-forming period and the longer the period of acceleration. The poorer the condition the shorter the periods.

I have chosen the reciprocal curve, for in this form it becomes quite clear that it is identical with Fig. 1, showing increase in size of eggs, and decreased per cent. of jelly layers, with age. I will discuss this correlation later.

There is one other matter that deserves brief mention. It was observed repeatedly that physiologically good eggs form membranes a considerable distance from the egg surface. With increasing age and with change in rate of membrane formation, the membranes are formed closer and closer to the egg, until they could barely be distinguished from the plasma membrane, and finally did not appear at all. With this reduction in width of the perivitelline space there appeared to be a greater tendency toward scalloping or blistering in the early appearance of the membrane. The Hertwigs in '86 observed a similar narrow perivitelline space and scalloping in ageing eggs.

Arbacia.

Some observations upon *Arbacia* eggs gave similar results, with one exception, namely, there was no initial acceleration of rate in membrane formation so characteristic of *Toxopneustes*. But this lack of acceleration may however have been due to insufficient early observations. The facts are brought together in Table II.*b*.

In experiment 1 (Table II.*b*), the eggs of 2 out of the 5 females that formed membranes at all, after the first observations, showed retardation in rate with age.

In experiment 3, the eggs of 4 out of the 7 females that formed membranes after the first observation, also showed retarded rate of membrane formation.

In experiment 2, one female showed retardation, another

showed no change in rate and the other 4 did not form membranes after the first observation.

At this point the question arose whether the inability to form membranes was due to the sperm or to the egg or to both. The matter was tested as follows: *after eggs and sperm of the same age no longer formed membranes*, the eggs were fertilized by fresh sperm, and vice versa, freshly liberated eggs were tested with old sperm. In the first case, *i. e.*, stale eggs fertilized by fresh sperm, fertilization membranes reappeared, and were formed in most instances as rapidly as in previous matings. For example, in experiment 3, membranes ceased to appear when both germ cells were 23 hours old. When 24 hours old, the eggs were fertilized by 4-hour-old sperm, and membranes appeared in 3 out of 7 females. The eggs of 2 females formed membranes in 2½ minutes each, which was more rapid than when the eggs were 18½ hours old. The third formed membranes in 6 minutes. These observations showed that the first inability to form membranes was not due to the absence of membrane-forming substance in the eggs.

When fresh sperm was used with eggs 42, 48, 65 and 70 hours old, no membranes were formed. This showed that at this age the lack of membranes was now due to a change in the eggs which began about 24 hours after liberation in some females, and in 42 hours in other females. The membrane substance was gone.

In the reverse experiments, when increasingly old sperm was used to fertilize fresh eggs, membranes were formed as rapidly and as extended from the surface of the egg as when fresh sperm was used (within certain limitations). For example in experiment 4, 23-hour-old sperm caused membranes to appear in 7, 3, 3, 3 minutes in the 4 females. In experiment 5 the sperm which was 45½ hours old formed membranes in 2 of the 3 females in 2 and 2½ minutes respectively. In experiment 6, when sperm was 73 hours old, membranes appeared in 2½, 4, 4½ and 5 minutes respectively. Even when sperm was 95 hours old (experiment 6) one out of 4 females formed membranes in 2½ minutes.

These and other facts clearly show that *the rate and charac-*

ter of membrane formation, in aging eggs, are functions largely of the egg and determined by their physiologic condition at liberation and by their subsequent age. Physiologically good eggs form clear membranes rapidly with either fresh or old sperm (the maximum age of such sperm was not ascertained). Physiologically poor eggs, form membranes more slowly, closer to the egg surface or do not form membranes at all with either fresh or stale sperm. The change in rate of membrane formation with age, constitutes the third index of the physiologic deterioration of the eggs with age.

Effect of Age Upon Cleavage.

Synchronous Aging of Germ Cells.

Aging or physiologic deterioration may be measured either (1) by a change in size, or (2) a loss of the jelly layer, or (3) a change in rate and character of membrane formation. There is another, more exact and more finely graded index of the changes in the germ cell with age, namely, (4) changes in cleavage.

Hertwig, '96, and Loeb suggested that with age there was a decreasing cleavage. F. R. Lillie, '14, showed in much greater detail that there was a decrease and indicated the extent of the decrease.

In Study I., I showed that under constant and optimum conditions freshly liberated eggs from different freshly collected females frequently differed widely in the total per cent. of cleavage in a given time, even when fertilized by the same male. In *Toxopneustes* the different females vary from 11 to 87 per cent., in *Hipponoë* from 5 to 81 per cent., in *Arbacia* from 0 to 90 per cent. Such differences were ascribed to differences in physiologic condition of the eggs of the different females at the time of liberation.

In preliminary experiments, a given pair of tested sea urchins were used for successive fertilizations. From 100 to 200 of the fertilized eggs were examined at each interval, and the sperm used was freshly prepared from the dry sperm.

In one group of experiments there was an unmistakeable direct reduction in the total cleavage with age. For example, in experiment 1, the eggs of 3 females were fertilized by one male when

the germ cells were from ¼ to 6 hours old. The per cent. of the total number of eggs that cleaved at each interval was 98 per cent. when 17 minutes old, 80 per cent. when 42 minutes old, 70 per cent. when 98 minutes old, 33 per cent. when 161 minutes old, 40 per cent. when 214 minutes old, 18 per cent. when 294 minutes old, 0 per cent. when 360 minutes old. The other 2 females showed corresponding decreases. The average for the 3 females was 94, 62, 54, 28, 31, 11 and 0 per cent. respectively. See Table III.

In the other group of experiments, there was an initial increased cleavage in the eggs of some or all of the females, followed by a definite and progressive decrease. For example in experiment 6, the eggs of female 1 and 4 decreased in cleavage with age, as in the first group. But the eggs of females 2 and 3 increased in cleavage for a brief period after the first observation, and then the per cent. decreased. This appeared to me at the time to be an error in observation, but similar increments occurred in other experiments. In experiment 7 the eggs were fertilized at early and frequent intervals, namely, 7, 20, 43, 59, 89 and 140 minutes. All 5 series of mating showed an early *progressive increased cleavage*, followed after 1 hour, by a decrease. The average for all 5 series was 49, 66, 88, 62, 40 and 25 per cent.

It is probable then that in the other experiments the apparent absence of an initial increased cleavage was due to failure to make sufficiently early and sufficiently frequent intervals, or possibly that the increase in cleavage becomes evident only in eggs in poor physiologic condition.

In *Arbacia* only the direct decreased cleavage was observed. Whether an initial increase occurred I cannot say. In the first place insufficient early observations were made, and in the second place, with physiologically good eggs, the maximum or nearly maximum cleavage took place, so that any physiologic change could not be manifested by an increased cleavage.

Only two experiments are given to illustrate the behavior of aging eggs of *Arbacia*. In experiment 8, the eggs of five females fertilized by one male, averaged 80 per cent. when 4 hours old, 33 per cent. when 23 hours old, 0 per cent. when 28½ hours old. (See Table III.)

TABLE III.

SHOWS CHANGE IN TOTAL CLEAVAGE WITH PROGRESSIVE AGE OF GERM CELLS. *Toxopneustes* AND *Arbacia.*

Toxopnuestes.

No.	Date.	Age of Germ Cells in Minutes.	Per Cent. of Eggs that Cleaved.							Time After Fertilization, Minutes.	Average Per Cent. Cleavage.	Per Cent. Loss per Hr.
			Females 1.	2.	3.	4.	5.	6.	7.			
1	7/12	17	98	98	87					120	94	
		42	80	76	31						62	
		99	70	72	20						54	
		161	33	38	14						28	
		214	40	30	23						31	
		294	18	14	2						11	
		360	0	0	0						0	18.0
2	7/19	130	82[1]	96	83	96	98	94		120	91	
		240	93	99	84	89	93	91			91	
		350	92	98	82	64	81	12			71	
		470	77	64	70	4	82	10			51	7.0
3	7/16	160	50	74	81	96	18[1]	14	40	120	53	
		300	34	61	37	92	34	0	50		40	
		500	28	8	20	29	1	6	25		16	6.5
4	7/21	120	51	98						120	74	
		323	11	84							47	8.0
		450	0	0							0	
5	7/14	20	92	47	98					120	79	
		80	99	95	97						97	
		140	98	95	97						96	
		210	96	93	97						95	
		280	92	82	87						87	
		370	62	69	85						72	
		440	82	59	87						76	
		500	43	10	36						29	8.2
		810	0	0	0						0	
6	7/7	75	91	41	67	86				120	71	
		84	75	87	98	80					85	
		180	80	80	17	60					59	7.2
7	7/3	7	80	23	35	68	43			113	49	
		20	95	46	75	73	42			105	66	
		43	80	85	88	95	95			87	88	
		59	62	71	76	19	86			91	62	
		89	38	50	73	31	11			91	40	
		140	34	12	9	11	11			85	25	

Arbacia.

8	8/12	4 hrs.	95	97	18	95	95			60	80	
		23 "	57	49	0	0	60			67	33	2.5
		28½ "	0	0	0	0	0			60	0	
9	8/16	1½ "	100	98	100	88	98	92	100	64	96	
		18½ "	75	20	95	62	88	55	51	60	63	1.8
		23 "	0	0	0	0	0	0	0	60	0	

[1] Probably an error in recording.

Fig. 1 represents schematically the change in rate of cleavage with age.

The per cent. cleavage is a convenient and finely graded index not only of the physiologic condition of the eggs at the time of liberation, but at subsequent intervals. It became then a simple matter to compute the rate of deterioration with age for a given female, or group of females. The rate of deterioration, *i. e.*, the reduction in cleavage, in per cent. per hour, varied from 6.5 per cent. per hour in experiment 3, to 18.0 per cent. in experiment 1, viz: 6.5, 7.0, 7.2, 8.0, 8.2 and 18 per cent. per hour, for nearly comparable periods. These rates are strikingly greater in *Toxopneustes* (from tropical waters) than in *Arbacia* (of the temperate waters of Massachusetts). Experiments 8 and 9 are illustrative of the rate of deterioration in *Arbacia*, viz: 1.8 per cent. per hour, in experiment 9, 2.5 per cent. per hour, experiment 8. When it is recalled that the difference in temperature of the waters at the two laboratories is about 10° C., and the difference in physiologic deterioration 3 to 10 times as great, it must be evident that the difference in rate is conditioned not only by temperature but by differences in HO concentration, and the egg protoplasm of the different species.

The above data did not make clear whether the decreased cleavage was due to the deterioration of the eggs or of the sperm or both sperm and eggs. The answer to these questions could be obtained in two ways: First to fertilize increasingly old eggs each time with freshly liberated and tested sperm, which would test the rate and degree of degeneration of the eggs. Secondly to fertilize freshly liberated and physiologically good eggs with fresh suspensions of increasingly old sperm, and test the degeneration of the sperm. Both series of experiments were made.

Effect of Age Upon Eggs.

Aging Eggs fertilized by Fresh Sperm. Longevity of Eggs. Toxopneustes.

In the following experiments, eggs and sperm aged synchronously until the eggs either no longer cleaved or only a very small per cent. cleaved. Then samples of the same eggs were fertilized at each subsequent interval by freshly liberated sperm. See Table IV.

In experiment 1, for example, the eggs of 4 females fertilized

TABLE IV.

SHOWS CHANGE IN CLEAVAGE WHEN PROGRESSIVELY AGING EGGS ARE FERTILIZED BY SPERM OF VARYING AGES. *Toxopneustes.*

No.	Date.	Age of Germ Cells in Minutes.		Per Cent. of Cleavage Eggs.						Time in Minutes.	Average Cleavage Per Cent.
		♀.	♂.	Female 1.	2.	3.	4.	5.	6.		
1	7/2	64	64	0	1	0	1			120	½
		209	19	8	85	70	10				43
		250	60	24	80	50	26				45
		309	120	3	44	14	17?				19
		400	3	8	70	2	10				22
2	7/12	420	420	6	2	0				120	3
		436	60	98	92	60					83
3	7/14	500	500	43	10	36				120	29
		600	2	50	36	81					55
		720	720	0	0	0					0
		10	600	33	0	0	10				10
4	7/19a	660	660	0	0	0	0	0	0	120	0
		660	90	95	94	13	32				59
		675	105	10	50	10	12	10	7		16
5	7/19b										
	a)	610	610	0	4					120	
				0	0						
				0	4						1
	b)	640	60	80	8						
				36	50						
				70	25						45
	c)	20	600	47	33	4	23				25
	d)	30	30	18	44	22					28
6	7/16	660	660	5	5					120	5
		7	660					91			91
		670	7	69	54	78					
				78	78						
				40							
		7	7				100				100
7	6/21	660	660	0						120	0
		660	20	18							18
		660	20	13							13
		20	660	0	0						0
		20	20	0	0						0

by the one male, gave a decreasing per cent. of cleavage with age, until the germ cells were 64 minutes old, when only 0, 1, 0 and 1 per cent. of the eggs of each of the females cleaved. The eggs were allowed to age further until they were 209 minutes old, when they were fertilized by freshly liberated sperm, and cleavage rose to 8, 85, 70 and 10 per cent. respectively. This was 42½ per cent. greater than the previous observation. It was clear that the failure to cleave when 64 minutes old was due to the almost complete deterioration of the sperm. It is not clear from this experiment how much the eggs have deteriorated. For

as I have shown the results obtained by sperm from different individuals may not be compared without further testing. In this experiment, when the eggs were 225 minutes old, and sperm only 35 minutes old, 32 per cent. cleaved. When the eggs were 250 minutes old and the sperm 60 minutes old, the average cleavage was 45 per cent. Even when the eggs were 400 minutes old, and fertilized by freshly liberated sperm, 22 per cent. cleaved. Hence it is clear that the decline to the zero mark when both germ cells were 64 minutes old, was due not to the death or incompatibility of the eggs, but of the sperm.

Even more striking results were obtained in other experiments. In experiment 2, for example, cleavage decreased progressively until the germ cells were 420 minutes old, and 6, 2 and 0 per cent. of the eggs cleaved. When the eggs 16 minutes later, *i. e.*, 7¼ hours old, were fertilized by sperm only 1 hour old, the cleavage was 98, 92 and 60 per cent. or an increase of 80 per cent. over the last reading. When the eggs of these same females were liberated, and immediately fertilized they averaged 80 per cent. hence it is probable that very little real deterioration of the eggs occurred during the first 7 hours after liberation.

In experiment 3, when the germ cells were both 8 hours old, the average cleavage was 29 per cent., when 12 hours old 0 per cent. When the eggs were 10 hours old and the sperm 2 minutes old, the average cleavage rose to 55 per cent.

In experiment 4, 11-hour-old germ cells gave 10 per cent. cleavage. Eleven-hour-old eggs by moderately fresh sperm gave 59 per cent. cleavage. Fifteen minutes later using the same eggs and sperm, only 16 per cent. cleaved. The marked reduction was due again to the rapid deterioration of the sperm of the second male.

In experiment 5, the eggs of 2 females were tested by 3 different males. When the germ cells were 5 hours old 1 per cent. cleaved. When tested by moderately fresh sperm, cleavage rose to 45 per cent.

In experiment 6, the increased cleavage with fresh sperm was 57 to 73 per cent.

In experiment 7, the small increase with fresh sperm, *i. e.*, only 18 per cent. and 13 per cent. respectively, was due to the

poor physiologic condition of the sperm as determined by tests with fresh eggs.

In every experiment, freshly liberated sperm raised the total cleavage from 0 or nearly 0 to 30, 40, 50 and even 80 per cent. of the eggs examined. There can be little doubt but that this means that the apparent death of the eggs (when no cleavage occurred) was really due to the precocious and rapid deterioration of the sperm, with moderately little deterioration of the eggs.

Hence to determine the exact physiologic condition of the eggs at any stage in the ageing cycle, and to determine the real longevity of the eggs, it is necessary to fertilize the eggs with freshly liberated sperm at each testing, as F. R. Lillie, '14, had done for a very different purpose. For example, the eggs of 4 females of experiment 1, Table V., when ⅓ hour old, gave 95

TABLE V.

SHOWS REDUCTION IN CLEAVAGE WHEN AGEING EGGS ARE FERTILIZED BY FRESH SPERM. SHOWS TRUE LONGEVITY OF EGGS. *Toxopneustes.*

No.	Date.	Age of Germ Cells in Hours.		No. of ♂.	Female No.						Average Cleavage, Per Cent.
		♀.	♂.		1.	2.	3.	4.	5.	6.	
1	7/4	⅓	⅓	1	95						95
		20	⅓	2	50	43	95	80			67
		23	1⅔	2	0	4	0	3			2
		24	1/10	4	0	13	0	0			3
		25	1	4	0	1	2	0			1
		46	1	5	0	2	0	0			½
		48	1/10	6	0	.0	0	0			0
2	7/5	23	1/10	1	7	0	0				1
		25	1	2	45	0	2				—
		50	1/10	1	0	0	0				0
3	7/7	46	⅓	1	6	0	1	0			1¾
		46	¼	2	0	0	1	0			¼
		47	¾	3	0	2	1	1			1
4	7/8	½	½	1					90	81	85
		23	⅓	1	0	22	43	25			22
		51	1/10	2	0	0	0	0			0

per cent. average cleavage; when 20 hours old, tested by fresh sperm, 67 per cent.; when 23 hours old, 7 per cent.; when 24 hours old, 3 per cent.; when 25 hours old, 1 per cent.; when 46 hours old, ½ per cent.; when 48 hours old, 0 per cent. These figures give a much more exact measure of the rate of deterioration of the eggs than when synchronously aged eggs and sperm

were used. There was very little deterioration or dying until the 20th hour, and then a most rapid rate of destruction until the 24th hour, and a final residual minimum extending until the 48th hour. The eggs of female 1 died at 23 hours female 4 at 24 hours, female 3 at 25 hours, female 2 at 48 hours.

Similar results were obtained in experiments 2, 3 and 4. Forty-eight hours appeared to be the maximum longevity of the eggs of *Toxopneustes* under the given experimental conditions. Table V. gives the rate of deterioration of the eggs, Table III. of the sperm.

Arbacia.

The experiments were repeated with *Arbacia* eggs, and the inquiry was pushed further. The data are brought together in Table VI. The facts may be summarized as follows:

1. Eggs which no longer cleaved when fertilized by sperm of the same age, did cleave when fertilized by freshly liberated sperm. In experiment 1, the increase was from 0 to 39 per cent.; in experiment 2, from 0 to 56 per cent.; in experiment 3, from 0 to 44 per cent., etc. This is exactly as in *Toxopneustes*.

2. With further aging there occurred a second cycle of progressively decreasing cleavage. For example in experiment 5 24-hour eggs fertilized by 1-hour sperm averaged for the 6 females 42 per cent. When the same germ cells were 31 and 8 hours respectively, they averaged only 10 per cent. A similar secondary deterioration occurred in experiment 1. The rapid deterioration was due in both groups to the more rapid destruction of the sperm than of the eggs, as in *Toxopneustes*.

3. The real deterioration of the *eggs* was determined as in *Toxopneustes* by fertilizing aging eggs with freshly liberated sperm at each testing. It was found that the eggs were very long lived. In experiment 7, 20-hour eggs showed little deterioration, 96 per cent. cleaved. Even when 41 hours, there was little deterioration, for 81 per cent. cleaved. When 48 hours 19 per cent., when 65 hours 9 per cent., when 72 hours 1 per cent. The other experiments, viz: 2, 3, 5, 6, 7, 8, 9, 10, 11, etc., gave similar results. They showed the same characteristics as *Toxopneustes* eggs, namely, *a long period of little deterioration, which in Arbacia is about 20 hour under the given experimental*

TABLE VI.

SHOWS REDUCTION IN CLEAVAGE WHEN AGING EGGS ARE FERTILIZED BY FRESH SPERM. SHOWS LONGEVITY OF EGGS OF *Arbacia*.

No.	Date.	Age of Germ Cells in Hours.		Female Number.							Time, Minutes.	Average Cleavage, Per Cent.	Loss Per Cent. per Hr.
		♀.	♂.	1.	2.	3.	4.	5.	6.	7.			
1	8/12a	4	4								60	80*	
		28½	28½	0	0	0	0				60	0	
		28½	4½	29	33	28	67				60	39	
		48	½	2	½	0	0				60	1	1.8
		1½	1½								60	96	
2	8/16	23	23	0	0	0	0	0	0	0	60	0	
		24	4	81	79	82	0	65	82	8	60	56	
		42	1	30	0	80	0	0	0	0	90	15	
		42	⅛	77	0	72	0	0	0	0	60	21	
		48	1/10	90	3	79	0	0	0	0	90	24	
		65	¾	48	0	0	0	0	0	0	60	7	
		70	1/12	21	0	0	0	0	0	0	60	3	1.3
		88	1/12	0	0	0	0	0	0	0	60	0	
3	8/17	1½	1½	96	70	97					120	87	
		18½	18½	71	20	93						61	
		23	23	0	0	0						0	
		24	4	28	50	65						44	
		42	1	30	0	17						15	
		42	1/10	66	0	70						45	
		48	1/0	52	3	74						43	
		65	¼	45	4	1						16	
		70	1/12	18	6	5						9	1.1
4	8/17	46	1/12	40	41	67					60	43	
		7	25	0	0	0						0	
5	8/13	4	5	98	97	99	99	98	93		120	97	
		24	1	87	3	57	89	19	0			42	
		31	8	64	0	0	0	0	0			10	3.2
		49	1¼	0	0	0	0	0	0			0	
		49	1¼	0	0	0	0	0	0			0	
6	7/29	20	1/12	73	85						120	79	
		43		34	35							34	
		51		7	5							6	2.3
7	8/1	20–22	1/12	96	96						120	96	
		41		75	87							81	
		48		14	24							19	
		65		3	15							9	
		72		2									1.8
8	8/3	41	1/12	11	22						120	16	
		63		8								8	
		87		0								0	
9	8/9	48	1/12	9	36						120	22	
		68		0								0	
10	8/10	45	1/12	2	7						120	4	
		49		1	7							4	
		51		0	0							0	
		69		0	0							0	
11	8/11	20	1/12	89								89	
		40		7								7	

* Much cleavage, exact count not made.

Table VI.—*Continued.*

No.	Date	Age of Germ Cells in Hours.		Female Number.							Time, Minutes.	Average Cleavage, Per Cent.	Loss Per Cent. per Hr.
		♀4.	♂4.	1.	2.	3.	4.	5.	6.	7.			
12	8/18	6	28	49	99	95	99				90	85	
		1	45½	0	0	0					60		
				91	47	97					120	81	
		5	49½	0	17	85					60		
				93	31	77					120	67	
		1/10	68	39	90	52	48				60		
				100	100	83	40				120	80	
		5	73	66	74	75	45				60		
				90	69	87	100				120	86	
		10	77	*	*	*							
		1/10	95	29	59	72	78				275	59	
13	8/12a	48	48	14							60		
				14							120	14	
	b	18	48	80							60		
				98							120	98	
	c	1/2	48	60							60		
				70							120	70	
	d	48	18	11							60		
				13							120	13	
	e	18	18	89							60		
				92							120	92	
	f	1/2	18	58							60		
				70							120	70	

conditions. Then there occurred a period of rapid deterioration, i. e., until the 40th hour, and thirdly a long period of minimum cleavage ranging from 50th to the 70th hour. Arbacia differs only in the rate of deterioration, which is several times slower than in *Toxopneustes.*

4. The longevity of *Arbacia* eggs was correspondingly longer than *Toxopneustes.* In experiment 1, the eggs of 2 out of 4 females ceased to cleave when 48 hours old. In experiment 2, the eggs of 4 females ceased to cleave at 42 hours; the fifth female at 65 hours, and the 6th female at 88 hours. In experiment 3, cleavage occurred in all 3 females at least till the 70th hour, etc., etc. The longevity ranged from 24 hours to beyond 72 hours, or about twice that of *Toxopneustes* eggs, due largely to the difference in temperature (about 10° C.) of the sea water.

Effect of Age Upon Sperm.

Freshly Liberated Eggs Fertilized by Aging Sperm. Longevity of Sperm.

In the reciprocal experiments, after the synchronously aging germ cells, no longer cleaved, the aging sperm were tested against freshly liberated eggs.

In experiment 3 (Table IV.), when the *Toxopneustes* germ cells were both 8 hours old, 29 per cent. of the eggs cleaved; when 12 hours old 0 per cent. cleaved. When the sperm were 10 hours old, they were tested by fresh eggs, 10 minutes old, and gave an average cleavage of 10 per cent. This might suggest that the rate of deterioration in synchronously aging sperm cells was conditioned by the rate of deterioration of the sperm.

In experiment 5, 10–hour–old eggs fertilized by 10-hour-old sperm averaged 1 per cent. cleavage. When the sperm was tested by fresh eggs, the cleavage rose to 25 per cent.

Similarly in experiment 6, 11-hour old germ cells averaged but 5 per cent. cleavage; the old sperm by fresh eggs gave 91 per cent. cleavage. On the other hand, in experiment 7, 11-hour-old germ cells gave 0 per cent. cleavage, in both crosses.

The explanation for these apparently contradictory data is found in a triple comparison, namely,

1. Crossing of synchronously aged germ cells.
2. Crossing of aged eggs by fresh sperm.
3. Crossing of fresh eggs by aging sperm.

If this be done it becomes apparent at once that the results are due in largest part to the condition of the egg, subject only to the ability of the sperm no matter how deteriorated to penetrate the cortical layer and initiate development.

In experiment 5, Table IV., 10-hour-old germ cells gave 1 per cent. average cleavage. When the eggs 10½ hours old were fertilized by moderately fresh sperm, 45 per cent. cleaved; the reciprocal cross, viz,, fresh eggs by the old sperm, caused only 25 per cent. to cleave. When these fresh eggs were tested by fresh sperm, 28 per cent. cleaved, *i. e., approximately the same cleavage as when fertilized by 10-hour-old sperm.* Hence low cleavage in experiment *a* was due to the sperm, while in *c* and *d* it was due to the eggs.

In experiment 6, similar results were obtained. Eleven-hour-old germ cells gave 5 per cent. cleavage. Fresh eggs used to test the old sperm gave *91* per cent. cleavage. But when these fresh eggs were tested by fresh sperm, *approximately the same percentage cleaved as when fertilized by stale sperm.*

Experiment 7 is interesting because the fresh eggs used were in

physiologically poor condition, and when fertilized by 11-hour-old sperm gave 0 per cent. cleavage. These same eggs tested by freshly liberated sperm gave the same results, namely 0 per cent.

Hence the results obtained by fertilizing fresh eggs by old sperm is determined in largest part by the physiologic condition of the eggs. The sperm plays but a minor rôle. One must be cautious, however, in any given experiment, in interpretng the results. In experiment 3, for example, the 10-hour-old sperm gave only 10 per cent. cleavage with fresh eggs. This low cleavage might have been due to the physiologically poor condition of the sperm or of the eggs. Actual tests showed that the fresh eggs were in poor condition and not the old sperm.

If the sperm deterioration is not reflected, or only to a very slight degree reflected, in a reduction in cleavage how does it happen that in all experiments *when aged eggs were fertilized by aged sperm the cleavage was far less than in either of the other two crosses.*

This result is probably due to a summation of effects, that due to the deteriorated condition of the eggs, and that due to the probable greater difficulty of aged sperm to initiate mitosis and cleavage. In the final analysis both are reducible to the condition of the egg.

Arbacia.

Further light was thrown upon these questions by the experiments with *Arbacia*.

Experiment 12, Table VI., is illustrative of the effect of aging upon the sperm. In this experiment after the germ cells no longer cleaved, the old sperm was tested against fresh eggs of 4 different females, over a long range of intervals. When the sperm were 28 hours old, the eggs of the 4 females averaged 85 per cent. cleavage (90 minutes after fertilization). When the sperm were 45½ hours old 81 per cent. of the fresh eggs of 4 other females cleaved; *when 73 hours old 86 per cent.; when 77 hours old "very many" (exact count not taken); when 95 hours old 59 per cent. of the eggs cleaved.* At this last observation the different females gave 29, 59, 72 and 78 per cent. cleavage. How much longer the sperm could have fertilized freshly liberated eggs was not determined. How much of the reduction (when the sperm

were 95 hours old) was due to a physiologic deterioration of the sperm, comparable with that in the eggs, and how much the reduction was due to an excessive mortality, as a result of which there were insufficient numbers of effective sperm, was not determined. Both factors probably play important rôles, though I am inclined to believe that a differential mortality plays a major rôle.

In the above experiment and in others, *the decrease in cleavage with these very old sperm was amazingly small, far less than in eggs of like age.* It should be noted however that the sperm in these experiments were allowed to age in the "dry" or concentrated condition, without the addition of sea water, and in this condition their metabolism is at a minimum, hence the maximum longevity.

In experiment 13, various types of crosses corroborate the above conclusions. Eggs and sperm were fertilized at varying intervals until both germ cells were 48 hours old, at which time only 14 per cent. cleaved. This 48-hour-old-sperm was tested with 18-hour-old eggs and with $\frac{1}{12}$-hour-old eggs. The 18-hour eggs gave 98 per cent. cleavage; the $\frac{1}{12}$-hour eggs 70 per cent. Samples of the same eggs were also fertilized by sperm 18 hours old. The 48-hour eggs gave 13 per cent. cleavage; the 18-hour eggs 92 per cent. and the $\frac{1}{12}$-hour eggs 70 per cent. It will be at once evident that the sperm at both ages gave a *remarkably similar cleavage per cent. with fresh, with moderately old and with old eggs, i. e.*, the sperm have undergone little if any physiologic deterioration between 18 and 48 hours after liberation. The 48-hour eggs gave 14 per cent. cleavage by 48-hour sperm and 13 per cent. with 18-hour-old sperm. The 18-hour-old eggs gave 98 per cent. cleavage with 48-hour-old sperm and 92 per cent. with 18-hour sperm. The $\frac{1}{12}$-hour-old eggs gave 70 per cent. cleavage with 48-hour-old sperm and the same per cent. with 18-hour-old sperm.

The cleavage was determined essentially or exclusively by the condition of the eggs.

The decrease in cleavage in the fresh eggs was due as in *Toxopneustes* experiment to the poorer physiologic condition of the eggs at the time of liberation. *Good fresh sperm cannot cause physiologically poor eggs to cleave to any greater extent than old*

sperm. Cleavage in physiologically good eggs is not decreased by old sperm.

From these data it will also be observed that the longevity of dry sperm was greater than the eggs in the given experimental conditions. In neither *Toxopneustes* nor in *Arbacia* was the maximum longevity of the sperm determined.

Effect of Age Upon Rate of Cleavage.

I have shown that the total number of eggs that cleaved in a given time after fertilization, decreased as the eggs aged. I propose now to show that there was, as F. R. Lillie, '14, indicated, a corresponding retardation of the rate of early cleavage, with increasing age of the egg; and in a later paper I propose to describe the irregular and abnormal character of cleavage in very aged eggs.

The rate of cleavage was ascertained by the total cleavage at three intervals, namely, (1) 40 minutes after fertilization, when the eggs first divided, (2) 1 hour after fertilization, when most and sometimes all the eggs had divided once, and (3) 2 hours after fertilization when all or very nearly all the eggs had divided at least once.

The data are brought together in Table VII.

In experiment 1, the eggs of 6 females were fertilized by one male, when the germ cells were 130, 240, 350 and 470 minutes old. A record was made at each interval, of the number of eggs that cleaved in 40, 60 and 120 minutes after each fertilization. In female no. 1, 54 per cent. cleaved within 40 minutes, when the germ cells were 130 minutes old; 37 per cent. cleaved in the same interval when 240 minutes old; 3 per cent. when 350 minutes old, 5 per cent. when 470 minutes old. *There was an undoubted decrease in the rate of first cleavage with age. The cleavage at the same ages 60 minutes after fertilization also showed a similar retardation with age of the germ cells,* for 78, 85, 51 and 59 per cent. respectively, cleaved. The total cleavage, namely 2 hours after fertilization, as I have shown before, also decreased with age. The corresponding figures were 82, 93, 92 and 77 per cent.

The average for all 6 females brings out forcibly the retardation and decrease in cleavage. The average for the germ cells 40

TABLE VII.

SHOWS CHANGE IN RATE OF CLEAVAGE WITH AGE OF GERM CELLS. THE FIGURES REPRESENT THE PER CENT. CLEAVAGE IN INDICATED TIME. *Toxopneustes.*

No.	Date.	Age of Germ Cells in Minutes.	Female 1.			2.			3.			4.			5.			6.			Average Per Cent.			Ratio of Cleav in 40 Min.: 120 Min.	Ratio of Cleav in 60 Min.: 120 Min.
			40 Min.	60 Min.	120 Min.	40 Min.	60 Min.	120 Min.	40 Min.	60 Min.	120 Min	40 Min.	60 Min.	120 Min.	40 Min.	60 Min.	120 Min	40 Min	60 Min.	120 Min.	40 Min.	60 Min.	120 Min.		
1	7/19	130	54	78	82	70	93	96	73	88	83	82	88	96	86	94	98	91	80	94	76	86	91	83	94
		240	37	85	93	83	99	99	46	56	84	67	76	89	78	78	93	82	67	91	65	76	91	71	83
		350	3	51	92	72	94	98	22	59	82	67	57	64	70	75	89	35	26	12	44	60	71	62	84
		470	5	59	77	17	46	64	16	43	70	0	0	4	64	75	82	1	6	80	27	39	51	53	76
2	7/12	17		98			98			87															
		42	56	77	80	34	78	76	18	30	31										36	62	62	58	100
		99	0	35	70	6	50	72	1	12	20										2	32	54	3	59
		161	4	15	33	6	18	38	1	11	14										3	14	28	10	50
		214	3	24	40	6	28	30	1	11	13										3	21	31	9	67
		294	3	14	18	½	17	14	0	0	2										1	10	11	9	90
		360	0	0	0	0	0	0	0	0	0										0	0	0	0	0
		420	0	0	0	0	0	0	0	0	0										0	0	0	0	0
3	7/14	20	½	93	92	0	32	61	17	98	98										5	74	83	6	89
		80	0	87	99	0	95	95	0	35	70										0	72	88	0	81
		140	2	91	98	2	95	95	2	99	97										1	95	96	1	98
		210	8	83	96	8	63	93	12	97	97										9	81	95	9	85
		280	6	92	92	32	77	82	5	86	87										14	85	87	16	97
		370	6	61	62	4	46	69	3	47	85										4	51	65	6	78
		440	0	25	62	0	20	59	0	51	87										0	32	69	0	46
		500	0	12	43	0	½	10	0	5	36										0	6	29	0	20
4	7/16	160	0	83[1]	50	0	74	74	0	26	81	0	28	96	0	20	18				0	38	53	0	
		300	0	39	34	0	60	61	0	54	37	0	34	92	0	38	34				0	45	49	0	
		500	3		28	0		8	5		20	4		29	1		66				2		26	7	

[1] Probably an error in recording.

minutes after fertilization was *76, 65, 44 and 27 per cent.* for the 4 different ages of the germ cells. The average cleavage 60 minutes after fertilization, for the same ages, was *86, 76, 60 and 39 per cent.* and the total cleavage, *i. e.*, 2 hours after fertilization, was 91, 91, 71 and 51 per cent. respectively.

These observations suggest an explanation for the decreasing total cleavage with aging germ cells. For the decreased total may conceivably be due to a retardation of the division process, or an inhibition of this process, or both.

Records taken three hours after fertilization showed that the 2-hour observation really was the total cleavage; that very little and usually no additional cleavage occurred. *Hence the decreased total cleavage with aging germ cells was not an apparent decrease due to retardation, but a real decrease by inhibition of cleavage.*

We are concerned then with two symptoms or independent evidences of changes in the eggs caused by their aging. On the one hand, an increasing number of eggs were prevented from developing and, on the other hand, those that do develop, do so with increasing difficulty (retardation).

The relation between retardation and reduction in cleavage may be expressed as follows: When germ cells were 130 minutes old, 83 per cent. of the total number of dividing eggs cleaved within 40 minutes after fertilization; when the germ cells were 240 minutes old a less number, namely 71 per cent. of the cleaving eggs, divided in the same time. When the germ cells were 350 and 470 minutes old, 62 and 53 per cent. of the segmented eggs cleaved in 40 minutes.

In experiment 2, germ cells in poor condition were tested at 8 intervals, between ¼ hour and 7 hours, and the rate of cleavage noted at each interval. The results are essentially in accord with those of experiment 1. The per cent. cleaving in 40 minutes after each fertilization was 36, 2, 3, 3, 1, 0, 0 per cent.; for 60 minutes after fertilization, the record was 62, 32, 14, 21, 10, 0, 0; and the total cleavage 2 hours after fertilization was 62, 54, 28, 31, 11, 0, 0 respectively.

Experiment 4 is also quite in accord with the previous ones.

Experiment 3 differs from the above only in the increasing total cleavage for a brief period, followed by decreasing cleavage

with further age. The rate of cleavage follows a similar cycle; *the rate of cleavage and the total cleavage increased with the age of the germ cells until they were 280 minutes old, after which the rate was retarded and the total cleavage declined.*

Arbacia.

With *Arbacia* eggs the rate of cleavage was ascertained by noting the per cent. of the eggs that reached the 4-cell stage in 60 minutes. The data are shown in Table VIII.

TABLE VIII.

SHOWS CHANGE IN RATE OF CLEAVAGE FROM THE PER CENT. OF EGGS THAT REACH THE 4-CELL STAGE IN 60 MINUTES. *Arbacia.*

No.	Date.	Age of Germ Cells in Hours. ♀.	Age of Germ Cells in Hours. ♂.	Female 1.	2.	3.	4.	5.	6.	7.	Time, Minutes.
1	8/12	4	4	24	0	0	0	3			60
		23	23	1	0	0	0	1			67
		28	4½	2	6	5	12	36			67
		24	1	1	0	0	18	1			
		30½	8	0	0	0	0	0			
2	8/16	1½	1½	80	81	90	30	80	65	93	60
		18½	18½	3	0	0	0	0	0	1	
		24	4	3	10	1	0	6	1	1	
		42	1/5	1	0	0	0	0	0	0	
		48	1 8/4	3	0	2	0	0	0	0	
		65	3/4	4	0	0	0	0	0	0	
		70	1/10	2	0	0	0	0	0	0	
		88	1/10	0	0	0	0	0	0	0	
3	8/14b	1½	1½	42	22	67	77	90			60
		5	5	35	3	88	26	40			
4	8/14a	4	5	0	1	0	19	36			60
5	8/18	1	23	0	1	0	0				87
		5	49	0	8	0					60
		1/10	68	0	6	0					60
		5	73	5	5	3					60

In *Arbacia* as in *Toxopneustes* the total cleavage decreased and the early division process was increasingly retarded with increasing age of the germ cells. In experiment 3, for example, the eggs of 5 females were tested when their germ cells were 1½ hours old, and 59 per cent. of the eggs reached the 4-cell stage in 1 hour. When the germ cells were 5 hours old only 38 per cent. reached the 4-cell stage in the same time. In experiment 1 and 2, older eggs were used. The results were even more striking and in entire accord with the preceding experiments.

Retardation is another symptom of the deterioration not of the sperm but of the eggs.

Correlation of Changes.

I have shown firstly that the freshly liberated eggs of freshly collected females of the same species varied considerably with respect to size, jelly layer, membrane formation and cleavage; secondly the range of variability for each of these types of changes was ascertained; thirdly, these changes were correlated; fourthly, by *these correlated phenomena one could accurately and conveniently ascertain the exact physiologic condition of the eggs of any female, at any time.*

Freshly liberated eggs from freshly collected females could be readily classified into physiologically "good," "poor" and "bad" eggs, on the basis of the correlated phenomena, as follows:

	Physiologically Good Eggs.	Physiologically Poor Eggs.	Physiologically Bad Eggs.
Size:	Norm for species.	Larger than norm.	Still larger or smaller than norm.
	Small deviation from norm.	Increasing deviation.	Still further deviation.
Jelly layer:	Maximum number with jelly layers 100–90 per cent.	Increasing loss of layer 96–70 per cent.	Further loss 70–40 per cent.
Membrane formation:	Formed within 2 minutes.	Increasingly retarded. 3–7 min.	None.
Cleavage:	Maximum number cleave 100–90 per cent.	Decreasing number 90–60 per cent.	Further decrease. 60–0.

Perhaps one example chosen at random may make the matter more definite. The freshly liberated eggs of *Arbacia* (Experiment 8–12) were practically normal in size, over 90 per cent. contained the jelly layer, fertilization membranes were formed in 1½ to 2 minutes, the cleavage was 95, 97, 96 and 94 per cent. in the eggs of 4 out of 5 females. The eggs of the fifth female were larger than the norm, less than 90 per cent. possessed jelly layers, no membranes were formed upon fertilization and only 18 per cent. cleaved. The eggs of the same females were tested by a second male, and gave the same size and jelly count, as before, and membranes appeared in 2, 2, 2½, 2½ minutes respectively. The cleavage count was 72, 69, 62 and 55 per cent. This retardation

in membrane formation and reduction in cleavage was clearly due to the poor physiologic condition of the sperm. The eggs of the fifth female were likewise fertilized by this male and showed the same size and jelly count as before, but no membrane appeared and only 4 per cent. cleaved. By these tests it was definitely determined that the eggs of the first four females were in good physiologic condition, those of the fifth female in poor condition.

By these tests it is possible to state accurately whether any sample of eggs are "good," "poor" or "bad," and to state exactly to what degree of physiologic deterioration such sample of eggs may have reached. The words "good," "poor" or "bad" now have a specific meaning, in terms of definite measurable changes in size, loss of jelly, rate of membrane formation and cleavage, which changes symptomize and measure definite physiologic and morphologic changes in the eggs.

The freshly liberated eggs whose physiologic condition was determined, subsequently changed or aged or overripened, with respect to the same categories, namely, size, jelly layer, membrane and cleavage.

I have shown in this paper exactly the extent to which each of these categories varied with the aging of the germ cells. I wish now to emphasize the fact that the changes are correlated.

These correlated changes, each of which measures the degree of physiologic deterioration with age, may be briefly summarized in the following table:

	Freshly Liberated or Good Eggs.	Moderately Stale or Aged Eggs.	Very Stale or Aged Eggs.
Size:	Little deviation from the norm.	Increasing deviation in a plus direction.	Further deviation in plus or minus direction.
Jelly layer:	Maximum or close to maximum.	Decreasing.	Further decrease.
Fertilization membrane:	Within 2 minutes. Wide membranes.	Increasingly retarded. Increasingly narrow.	None.
Cleavage:	Maximum rate. Maximum number.	Increasing retardation. Decreasing numbers.	Further retardation. Further decrease.

Whatever the physiologic condition of the eggs when liberated, they undergo with age an increase in size, a decrease in the number

possessing the jelly layer, a decrease in rate of membrane formation, and a decrease in cleavage, varying in degree with the condition of the eggs at the time of liberation. And one may predict the extent of the other changes from any known one or two. If size or membrane rate are known, one may predict very approximately the other symptoms of the physiologic condition of the eggs, such as jelly and cleavage, etc.

The discussion of these results will be postponed until further data will be presented, concerning other types of changes in very aged eggs. For the present I wish to draw attention to the following considerations.

1. The aging process or processes which are symptomized in the various changes described in this paper begin not with the liberation of the eggs, but upon their maturation, within the body of the mother. This was first suggested by Loeb, and I am in entire accord with his view. Hence it follows that chronologic age (time since liberation) affords but a poor idea of the real physiologic condition of the eggs either at liberation or at any interval thereafter.

2. Aging is a continuous process, beginning within the body and continuing (with somewhat accelerated rate) outside of the body, culminating ultimately in the death of the eggs.

3. Aging was manifested in a number of ways, any one of which may serve as an index of the physiologic condition, and the degree of deterioration. *The ensemble of the various indices forms a clear and unmistakeable measure of their condition and their deterioration.*

4. While no attempt has thus far been made to describe the nature of the chemico-physical processes involved in the aging of the eggs, they may nevertheless be accurately measured. It is now possible to measure very accurately the physiologic condition of the eggs at liberation, and at any interval of time thereafter, to measure accurately the rate of deterioration or senescence under given experimental conditions. It is also possible to measure the real longevity of the eggs or of the sperm.

5. These data, and those described in Part III., afford a basis for an understanding of the nature of the aging process, and of the means of controlling senescence of the germ cells.

The discussion of the results and a more complete bibliography are given in Part III.

Summary.

The variation in size of the eggs, the per cent. with jelly layers, the rate of membrane formation and the total cleavage were ascertained for large numbers of freshly liberated eggs from freshly collected females, examined at different periods of the breeding season.

Three species of sea urchins were studied in this way, namely, *Toxopneustes* and *Hipponoë* of tropical waters, and *Arbacia* of the North Atlantic.

Large variations from the norm were observed and measured in all four categories. These variations were interpreted as indices of the physiologic condition of the eggs of each female at the time of liberation.

With the physiologic condition of freshly liberated eggs of a given female known, experiments were then instituted to ascertain the nature and extent of the changes in the germ cells, as they aged, or became overripe, under given optimum laboratory conditions.

The following is a brief statement of the changes in such aging germ cells, viz., changes in size, jelly layer, membrane formation and cleavage.

Change in Size.

Freshly liberated eggs in good physiologic condition varied but slightly from the norm. For details see text.

As these eggs aged, their volume increased continuously, until they cytolized or fragmented, and became smaller than the norm.

Freshly liberated eggs in poor physiologic condition either enlarged but little with age, or were directly reduced in size by cytolysis or fragmentation.

The nature, extent, and rate of change in size, depends upon the physiologic condition of the eggs when freshly liberated. Whatever the physiologic condition of the eggs may be, their senescence or physiologic deterioration can be very accurately measured by the degree of enlargement or reduction in their size.

Essentially the same results were obtained in the other two species.

Change in Jelly Layer.

Practically all freshly liberated eggs in good physiologic condition possess a jelly layer. Those in poor condition have a correspondingly less per cent.

With age the jelly layer was lost in an increasing number of the eggs.

The rate of loss depended upon the condition of the eggs at the time of liberation (all other conditions remaining constant).

For fresh eggs in good physiologic condition, the rate of loss per hour was 0.81 and for equally fresh eggs in poor condition 2.65 or over 3 times as rapid.

Essentially similar results were obtained in all three species.

The loss of jelly layer was a second symptom and index of the extent and the rate of ageing or senescence of the eggs.

Change in Membrane.

Freshly liberated eggs in good physiologic condition formed fertilization membranes within two minutes. The rate depended partly upon the sperm but primarily upon the physiologic condition of the eggs.

With increasing age the time required to form the fertilization membranes was at first accelerated and later retarded. In very aged eggs no membranes were formed.

Freshly liberated eggs in poor physiologic condition showed direct retardation in the rate of membrane formation.

Aging eggs which no longer formed membranes when fertilized by old sperm could be made to form membranes with fresh sperm.

The rate of membrane formation is practically independent of the sperm. It is essentially determined by the condition of the egg.

As the eggs aged the membrane appeared closer and closer to the surface of the egg; it became thinner and ultimately none was formed.

These observations are essentially the same for all three species.

The rate and character of membrane formation affords a third means of measuring senescence in eggs.

Change in Cleavage.

When freshly liberated eggs of *Hipponoë* and *Toxopneustes* in good physiologic condition were fertilized under the given optimum conditions at successive ages the total cleavage increased for a time and subsequently decreased,[1] and in extreme ageing none of the eggs segmented.

Cleavage then is an additional index of the degree of senescence or physiologic deterioration of the eggs.

There were two series of experiments, one in which the eggs and sperm aged synchronously, and the other in which they aged asynchronously. In both series the rate of decrease in cleavage with age was several times greater in *Toxopneustes* and *Hipponoë* than in *Arbacia*. This difference in rate of senescence as in rate of membrane formation, loss of jelly and change in size is due largely to differences in temperature, as well as to differences in HO concentration of the sea water, and to protoplasmic differences of the eggs.

When both germ cells aged synchronously the apparent longevity was about 11 hours for *Toxopneustes* and about 28 hours in *Arbacia*.

In asynchronous matings a more definite idea was obtained of the changes in the egg alone and in the sperm alone. When freshly liberated sperm were used to fertilize the eggs (of a female) at varying ages, with the precautions indicated in the text, the eggs showed progressively decreasing per cent. of cleavage (in *Arbacia*), but the rate of decrease was very much slower than when both germ cells aged synchronously. The decrease may be divided into three periods, the first a period of small decrease (about the first 20 hours in *Arbacia*) the second, a period of rapid and large decrease (between the 20th and 40th hour), and the third, a period of small decrease (between the 40th and 80th hour in *Arbacia*).

Eggs in poor physiologic condition at the time of liberation deteriorated at a correspondingly greater rate than physiologically good eggs.

[1] Freshly liberated eggs, of freshly collected *Arbacia*, in good physiologic condition, gave a maximum or nearly maximum cleavage and with ageing there was a direct decreasing total.

The real longevity of the eggs depended upon the physiologic condition at time of liberation, the better, the longer lived, and vice versa.

The rate of cleavage was accelerated for a time and then retarded with age, more so in physiologically poor eggs, less in good eggs.

When ageing sperm fertilized freshly liberated eggs the decrease in cleavage was surprisingly small. Very little decrease occurred when sperm were 73 hours, only a little more when sperm were 95 hours old.

The explanation for the greater longevity of the sperm is found in the fact that the sperm aged in the "dry" condition, in which they are inactive and hence minimum metabolism.

The greater decrease in cleavage in synchronously ageing germ cells is due to a summation of the injurious effects upon the sperm and upon the eggs.

In very late stages in ageing of sperm, the reduction may be due not so much to physiologic deterioration as to insufficient numbers of sperm. There is ground for belief that no matter how old or deteriorated the sperm if they are active (alive) they can penetrate the egg. It is not so clear whether such aged sperm cause parthenogenesis or sexual development.

The change in size, jelly, membrane and cleavage with aging of germ cells, are accurate, convenient and corroborative indices of chemico-physical and morphologic changes in the egg as they age, and afford convenient measures of the loss in vitality, or physical deterioration. And one change may serve for this purpose. Their ensemble is convincing.

In the next study will be considered the changes in much older germ cells leading towards their cytolysis and death.